

# Zebrafish and fly Nkx6 proteins have similar CNS expression patterns and regulate motoneuron formation

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Accepted 12 August 2004

Development 131, 5221-5232

Published by The Company of Biologists 2004

doi:10.1242/dev.01397

## Summary

Genes belonging to the Nkx, Gsh and Msx families are expressed in similar dorsoventral spatial domains of the insect and vertebrate central nervous system (CNS), suggesting the bilaterian ancestor used this genetic program during CNS development. We have investigated the significance of these similar expression patterns by testing whether Nkx6 proteins expressed in ventral CNS of zebrafish and flies have similar functions. In zebrafish, Nkx6.1 is expressed in early-born primary and later-born secondary motoneurons. In the absence of Nkx6.1, there are fewer secondary motoneurons and supernumerary

ventral interneurons, suggesting Nkx6.1 promotes motoneuron and suppresses interneuron formation. Overexpression of fish or fly *Nkx6* is sufficient to generate supernumerary motoneurons in both zebrafish and flies. These results suggest that one ancestral function of Nkx6 proteins was to promote motoneuron development.

Key words: *nkx6.1*, *Danio rerio*, *Drosophila melanogaster*, Hedgehog, Primary motoneurons, Secondary motoneurons, Interneurons, Eve, HB9, Islet

## Introduction

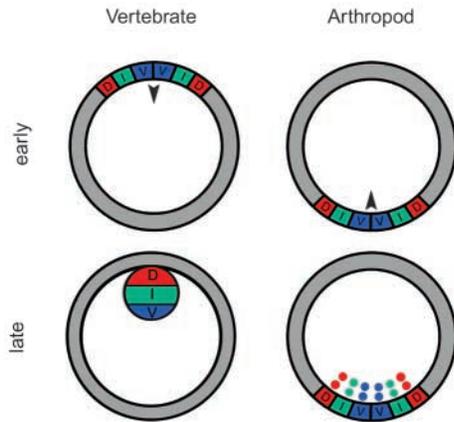
The growing number of genes expressed in similar patterns during axis formation and early central nervous system (CNS) patterning in arthropods and vertebrates has revived an old hypothesis (St.-Hilaire, 1822) postulating conservation of dorsoventral patterning in these phyla (reviewed by Sasai and De Robertis, 1997) (Arendt and Nubler-Jung, 1999; Cornell and Von Ohlen, 2000). Arthropods and vertebrates have been evolving away from a common ancestor for over 800 million years (Hedges, 2002) and their CNSs arise on opposite sides of the body, dorsal in vertebrates and ventral in arthropods (Fig. 1). Thus, it is not surprising that their CNSs are architecturally distinct, suggesting they are patterned by different mechanisms (Romer and Parsons, 1977). What is surprising is that despite these differences, corresponding spatial domains within the arthropod and vertebrate CNS express homologous genes (reviewed by Cornell and Von Ohlen, 2000).

The cell types that arise from corresponding CNS domains differ between vertebrates and flies. For example, in vertebrates all motoneurons arise from ventral CNS, whereas in flies motoneurons are generated from neuroblasts (NBs) at all dorsoventral levels (Bossing et al., 1996; Schmidt et al., 1999). Despite these differences, the arthropod and vertebrate neurectoderm exhibit similar patterns of gene expression. For example, fly neurectoderm expresses three transcription factor genes, *vnd*, *ind* and *msh*, in precise, longitudinal, ventral-to-dorsal domains (Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998). Mouse orthologs belonging respectively to

the Nkx, Gsh, and Msx gene families are similarly expressed in a ventral-to-dorsal orientation in longitudinal spinal cord domains, raising the possibility that these genes are a phylogenetically ancient mechanism to direct dorsoventral CNS patterning (reviewed by Cornell and Von Ohlen, 2000) (Arendt and Nubler-Jung, 1999).

Although these genes are expressed in strikingly similar spatial patterns in flies and mice, not all of them are expressed at corresponding developmental stages. All three fly genes are expressed early during CNS development (Chu et al., 1998; Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998), whereas only *Msx* genes are expressed at a corresponding stage in the mouse (Satokata and Maas, 1994; Wang et al., 1996). Thus, the hypothesis that similar mechanisms underlie dorsoventral patterning of arthropod and vertebrate neurectoderm predicts that vertebrates should have as yet uncharacterized, early-expressed paralogs of the fly genes.

Nkx genes are expressed in ventral spinal cord in mouse, chick and zebrafish. *Nkx2.2*, an ortholog of fly *vnd*, is required to establish a ventral interneuron precursor domain in the mouse (Briscoe et al., 1999). However, zebrafish *nkx2.2* is expressed relatively late in ventral spinal cord (Barth and Wilson, 1995), precluding involvement in early neural patterning. *Nkx6.1*, a family member that might fulfill an earlier patterning role has been described in the mouse and chick (Qiu et al., 1998). This gene is expressed early in medial neural plate and later in ventral spinal cord. Nkx6.1 is sufficient for motoneuron formation in the chick and required for formation



**Fig. 1.** Schematized morphogenesis of vertebrate and arthropod neurectoderm. Vertebrate and arthropod ectoderm viewed in cross-section (grey circles represent non-neural ectoderm). Vertebrate neurectoderm (colored boxes) is positioned dorsally (top of figure) on the body. Later neurectoderm undergoes morphogenesis (direction indicated by arrowhead) to form a neural tube, internal to the non-neural ectoderm. Dorsal CNS (D; red) is initially lateral, intermediate CNS (I; green) is initially intermediate and ventral CNS (V; blue) is initially medial. Arthropod neurectoderm (colored boxes) is positioned ventrally (bottom of figure) on the body. The nerve cord forms as neuroblasts bud off toward the interior of the embryo (direction of arrowhead). Later in development, the arthropod nerve cord contains layers of neurons. Early, members of the *vnd/Nkx* gene family are expressed in the ventral domain (blue) and members of the *msh/Msx* gene family are expressed in the dorsal (red) domain of both vertebrates and flies (see text for details). In flies, *ind* is expressed early in the intermediate domain. Although vertebrate *ind* homologs (*Gsh*) are expressed in a subset of intermediate domain cells, they are expressed early only in anterior CNS and much later in spinal cord (Deschet et al., 1998; Cheesman and Eisen, 2004; Valerius et al., 1995).

of a subset of motoneurons and interneurons in the mouse (Briscoe et al., 2000; Sander et al., 2000). However, a dramatic reduction in spinal motoneurons is only seen in mice lacking both *Nkx6.1* and a related gene, *Nkx6.2*, suggesting their functions overlap (Vallstedt et al., 2001). Flies also have an *Nkx6* gene that is expressed early in a subset of ventral neuroblasts and later in CNS neurons (Uhler et al., 2002). Thus, *Nkx6* genes in flies and vertebrates represent another pair of orthologs expressed in similar CNS domains.

To determine whether *Nkx6* genes play similar roles in specifying ventral CNS identity in vertebrates and arthropods, we investigated *Nkx6* function in zebrafish and flies. We cloned and characterized a zebrafish *nkx6.1* gene that is expressed initially in ventral CNS and later in motoneurons and some interneurons. Like other anamniote vertebrates, zebrafish have two distinct types of spinal motoneurons. Primary motoneurons (PMNs) are individually identifiable, born early, and have axons that pioneer motor nerves (Eisen et al., 1986). Secondary motoneurons (SMNs) are more numerous, born later (Myers, 1985), and have axons that follow primary motor axons (Pike et al., 1992). The ventral spinal cord domain that generates PMNs and SMNs also generates oligodendrocytes and at least three types of interneurons; Komer-Agduhr (KA), Ventral Longitudinal Descending (VeLD) and Circumferential Descending (CiD), each of which can be identified by soma

position and axonal trajectory (Appel et al., 2001; Bernhart et al., 1992; Park et al., 2002) (H. C. Park and B. Appel, personal communication).

Here we show that ectopic expression of *Nkx6* genes in zebrafish or flies results in embryos with supernumerary motoneurons, suggesting that at least one function of *Nkx6* genes has been preserved over the last 800 million years. Surprisingly, knocking down *Nkx6.1* protein in zebrafish reduces the number of SMNs, but not PMNs, raising the possibility of additional *nkx6* genes. *Nkx6.1* knockdown also increases the number of VeLD interneurons, suggesting that *Nkx6.1* protein regulates a decision between motoneuron and interneuron fate, consistent with our finding that there are fewer VeLDs and supernumerary motoneurons in embryos ectopically expressing *Nkx6.1*. Fly embryos ectopically expressing either fish or fly *Nkx6* show a similar phenotype.

The apparent conservation of *Nkx6* expression and function in flies and fish suggested these genes might be similarly regulated. As in other vertebrates, Hedgehog (Hh) signaling is necessary for expression of zebrafish *nkx6.1*. However, expression of fly *Nkx6* is unaffected by lack of Hh. These data suggest that although the signals establishing *Nkx6* expression have diverged, *Nkx6* proteins function as an ancient patterning mechanism to establish motoneurons within the CNS.

## Materials and methods

### Embryos

Zebrafish (*Danio rerio*) embryos were collected from natural crosses of adults, reared at 28.5°C, and staged according to hours postfertilization (hpf) and gross morphology (Kimmel et al., 1995). Wild-type embryos are genotype AB, the *syu<sup>td</sup>* allele is a deletion of the *shh* locus (Schauerte et al., 1998); *slow muscle omitted* (*smu*) encodes Smoothened, a component of the Hh signaling pathway (Chen et al., 2001; Varga et al., 2001) *GATA-2:GFP* fish are described by Meng et al. (Meng et al., 1997).

*Drosophila melanogaster* embryos were gathered from staged collections. Adults of the following genotypes were induced to lay embryos on a grape agar pad coated with yeast paste; *yw, sca>Nkx6* (fly gene), *sca>nkx6.1* (zebrafish gene), *eg>Nkx6* (fly gene), *w<sup>1118</sup>;hh<sup>ts2</sup> e/TM3 ftz lacZ, hh<sup>6n16e</sup>/TM3 ftz lacZ*.

### Zebrafish *nkx6.1* cDNA isolation

The following degenerate primers amplified an approximately 730 base fragment from 10 hpf cDNA (Ambion RETROScript Kit), forward 5'-TGCACTCCATGGCCGARATGAARAC-3', reverse 5'-CGCCGGTCTGGAACCANACYTT-3'. We designed specific primers and screened a gastrula stage cDNA library (Faucourt et al., 2001) to isolate a full-length clone; forward 5'-GCCTACCCGT-TATCTTCCACT-3', reverse 5'-GACTTGACTCTCTGTCTCGTTCCT-3'. Zebrafish *nkx6.1* GenBank Accession number is AY437556.

The Fly EST database (<http://flybase.bio.indiana.edu>) contains four identical clones with high similarity to the zebrafish *nkx6.1* homeodomain and NK domain, indicating the fly genome probably contains a single *Nkx6* gene, designated conceptual gene *CG13475*, also known as *Nk6* (Uhler et al., 2002).

Zebrafish *nkx6.1* and fly *Nkx6* (ResGen EST clone RE66661) ORFs trimmed of UTRs were inserted into the pCS2<sup>+</sup> MT expression vector (Rupp et al., 1994) for use in overexpression experiments.

### Phylogenetic analysis

MacVector software, utilizing the Clustal W algorithm, created a maximum likelihood tree. Sequences included in the tree were gathered from GenBank.

### In situ hybridization

Zebrafish RNA in situ hybridization was performed as described by Appel and Eisen (Appel and Eisen, 1998). RNA probes include *islet1* and *islet2* (Appel et al., 1995). RNA in situ hybridization on fly embryos was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989).

### Immunohistochemistry

#### Zebrafish

The following antibodies were used: monoclonal mouse anti-Islet (Korz et al., 1993) recognizes the Islet1 and Islet2 proteins (1:200; 39.4D5 Developmental Studies Hybridoma Bank), polyclonal rabbit anti-Nkx6.1 (1:1200; gift of O. Madsen), anti-Neurolin (1:4000) (Trevarrow et al., 1990) (also known as zn5 and DMGRASP; www.zfin.org), polyclonal anti-GABA (1:1000, Sigma), zn1 monoclonal (1:200) (Trevarrow et al., 1990) and znp1 monoclonal (1:1000) (Trevarrow et al., 1990). Embryos were processed as described by Appel et al. (Appel et al., 2001).

#### Flies

The following antibodies were used: mouse anti-Engrailed (1:5) (Patel et al., 1989) (Developmental Studies Hybridoma Bank), mouse anti-Eve (1:20; N. Patel), mouse anti-Eagle (1:500; M. Freeman and C. Doe), rabbit anti-Vnd (1:20) (McDonald et al., 1998), rabbit anti-Odd (1:100) (Spana and Doe, 1995), rat anti-Ind (1:250) (Weiss et al., 1998), mouse anti-FasII (1:100; C. Goodman), rat anti-Islet, guinea pig anti-HB9 (both 1:500) (Broihier and Skeath, 2002), rat anti-HB9 (1:500) (Odden et al., 2002), rabbit anti-pMAD (1:300) (Marques et al., 2002). Embryos were processed as described by Odden et al. (Odden et al., 2002).

### Microscopy

Images of zebrafish and fly embryos were captured on a Zeiss Axioplan equipped with a digital camera, or a Bio-Rad Radiance confocal microscope. Adobe Photoshop was used to adjust brightness and contrast of images.

### RNA and morpholino injections

*shh* mRNA (*shh* p64-T) (Krauss et al., 1993), *nkx6.1* mRNA (pCS2-MT), and *Nkx6* mRNA (pCS2-MT) were transcribed using the mMessage mMachine kit (Ambion) according to instructions. Two-cell stage embryos were injected with several nanoliters of either 0.1 mg/ml, 2 mg/ml and 2 mg/ml of *shh*, *nkx6.1* and *Nkx6* RNA, respectively. Numbers of PMNs were counted adjacent to somites 5-17 and subjected to a Student's *t*-test to determine significance.

To create zebrafish embryos with severely reduced Hh signaling, *tiggywinkle hedgehog* (*twhh*) and *echidna hedgehog* (*ehh*) morpholino antisense oligonucleotides (MOs) were injected as in Lewis and Eisen (Lewis and Eisen, 2001). An *nkx6.1* translation-blocking MO beginning at position -60 in the 5' UTR, (5'-CGCAAGAAGAAGG-ACAGTGACCCG-3') was designed by Gene Tools (Corvallis, Oregon). Several nanoliters of 2.5 mg/ml MO were injected as described by Lewis and Eisen (Lewis and Eisen, 2001). MO-injected embryos generally looked healthy but had little or no Nkx6.1 protein in the spinal cord at all stages assayed. Embryos injected with a 5-base mismatch MO (5'-CGgAAGAaAcGACAcTGAgCCG-3') had a wild-type pattern of Nkx6.1 protein in the CNS.

### Single cell labeling

Individual PMNs and VeLD interneurons were labeled using the methods of Eisen et al. (Eisen et al., 1989) and detected with an anti-fluorescein antibody (1:1000, Boehringer Mannheim).

### BrdU and TUNEL labeling

BrdU labeling was performed as described in Appel et al. (Appel et

al., 2001). TUNEL labeling was performed as described by Reyes et al. (Reyes et al., 2004).

### Generation of Fly UAS lines

A portion of fly *Nkx6* (EST clone RE66661, Research Genetics) and zebrafish *nkx6.1* sequences trimmed of UTRs were subcloned into the pUAS vector (Brand and Perrimon, 1993). Independent injection of these plasmids produced founding lines carrying the red eye marker (mini-white) that were outcrossed to *yw* flies. Transgenic progeny from this cross were crossed to the balancer lines *w;Gla/Cyo* and *w;TM3/TM6* to determine which chromosome carried the inserted transgene, and homozygous stocks were established.

### RNAi on fly embryos

Two non-overlapping ~500 base fragments of the Nkx6 coding region, excluding the homeodomain, were amplified by PCR for use as templates. RNA was injected as described by Sullivan et al. (Sullivan et al., 1999).

## Results

### Zebrafish and flies have Nkx6 genes

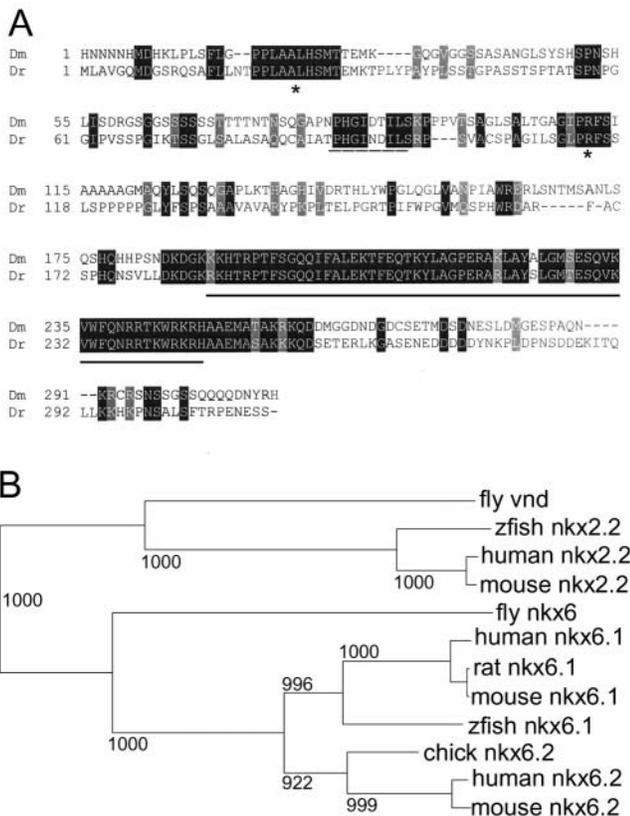
We used degenerate polymerase chain reaction (PCR) based on sequence homologies between human, mouse, rat and chicken Nkx6.1 proteins to identify a zebrafish *nkx6.1* gene fragment. Screening a gastrula stage cDNA library (Faucourt et al., 2001) isolated a longer clone with a complete open reading frame predicting a 312 amino acid protein containing an NK decapeptide, characteristic of the Nkx family and a homeodomain (Fig. 2A). The predicted zebrafish protein is closely related to other vertebrate Nkx6.1 proteins (Fig. 2B). Accordingly, we have named this zebrafish gene *nkx6.1*.

A BLAST search of the fly genome identified a conceptual gene, *CG13475*, for which there were four identical EST clones that match an *Nkx6* cDNA recently cloned from an embryonic fly cDNA library (Uhler et al., 2002). We confirmed flies possess a single *Nkx6* gene orthologous to vertebrate *Nkx6.1* and *Nkx6.2* genes (Fig. 2B). Comparing zebrafish and fly proteins, there is 93% amino acid sequence identity within the homeodomain and 80% identity within the NK decapeptide. Outside these two conserved motifs there are several other regions of high amino acid identity (Fig. 2A).

### Zebrafish *nkx6.1* is expressed in ventral spinal cord

To test our prediction that zebrafish *nkx6.1* would be expressed in ventral CNS, we performed RNA in situ hybridization. Transcripts were first detected at the onset of gastrulation in the embryonic shield epiblast (Fig. 3A). Near the end of gastrulation, *nkx6.1* was expressed in medial neurectoderm in two wide, diffuse stripes (Fig. 3B). By the 3-4 somite stage, *nkx6.1* was confined to a tight stripe in medial neural keel, extending from the midbrain through the posterior neural plate. This pattern was maintained throughout somitogenesis (Fig. 3D); Nkx6.1 expression persisted in ventral spinal cord until at least 48 hpf (data not shown). *nkx6.1* was detected in ventral hindbrain caudal to the midbrain-hindbrain boundary and in pancreas at later stages (Fig. 3C). To test for control at the level of translation, we labeled zebrafish embryos with a polyclonal anti-Nkx6.1 antibody designed against the highly conserved C-terminus of rat Nkx6.1 (Jensen et al., 1996). Although we cannot rule out the possibility that Nkx6.1 antibody crossreacts with additional Nkx6 proteins, at all stages examined protein

and RNA patterns appeared indistinguishable (data not shown). Cross-sections of 24 hpf embryos revealed Nkx6.1 expression in about five longitudinal cell rows in ventral spinal cord, including both medial and lateral floorplate (Fig. 4C); thus Nkx6.1-positive cells constitute approximately the ventral third of the spinal cord. This domain is similar to the *olig2* expression domain; *olig2* RNA is expressed in both progenitor and postmitotic cells (Park et al., 2002), thus, Nkx6.1 must also be expressed in both of these cell types. The neuroectodermal stripe of *nkx6.1* includes the domain in which motoneuron progenitors undergo their final division (Kimmel et al., 1994; Myers et al., 1986). To determine whether postmitotic motoneurons express Nkx6.1, we performed antibody double-label experiments. Islet (Appel et al., 1995; Korzh et al., 1993) and Nkx6.1 proteins are colocalized in PMNs during early somitogenesis (14 hpf, Fig. 4A); later Nkx6.1 protein is downregulated. By 18 hpf, Nkx6.1 and Islet proteins are largely mutually exclusive and Nkx6.1 is expressed only in a few PMNs (Fig. 4B). Cross-sections at 48 hpf revealed Nkx6.1-positive SMN nuclei surrounded by Neuroilin-positive plasma membranes (Fashena and Westerfield, 1999), indicating co-expression in these cells (Fig. 4D and data not shown). Thus both PMNs and SMNs express Nkx6.1 at least transiently.

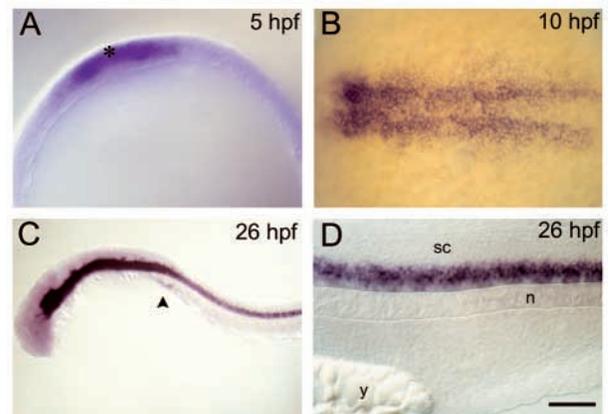


**Fig. 2.** Homology between Nkx6 proteins. (A) Alignment of fly and zebrafish Nkx6 translated amino acid sequence reveals several regions of high identity: the almost invariant NK domain (dashed line) and homeodomain (solid line), and two other regions indicated by asterisks. Fly Nkx6 was trimmed of the N-terminus. (B) Maximum likelihood tree. Fly Nkx6 is orthologous to vertebrate Nkx6 proteins, distinct from Vnd/Nkx2.2 proteins. Numbers at the nodes represent bootstrap values. Dm, *Drosophila melanogaster*; Dr, *Danio rerio*.

Recent fate-mapping revealed that in addition to motoneurons, several types of interneurons are derived from the *olig2*-positive ventral spinal cord domain (H. C. Park and B. Appel, personal communication). We tested whether one of these types of interneurons expressed Nkx6.1, by labeling individual VeLD interneurons with fluorescent dextrans, immediately fixing the embryos and examining whether the labeled cells co-expressed Nkx6.1. At 20 hpf most VeLDs were Nkx6.1-positive (Fig. 4E), however by ~24 hpf VeLDs had downregulated Nkx6.1 expression (Fig. 4F). Thus, we infer that VeLDs express Nkx6.1 early in development but downregulate it following axon extension.

### Fly *Nkx6* is expressed in ventral neuroblasts and motoneurons

*Nkx6* expression was first detected during early neurogenesis (stage 9) in the nerve cord midline, and it was weakly expressed in ventral column neuroectoderm of rostral segments (Fig. 5A). An hour later (early stage 10), *Nkx6* midline and neuroectoderm expression was downregulated. *Nkx6* expression was restricted to six ventral column neuroblasts, rostrally located in each hemisegment (Fig. 5B). Consistent with Uhler and colleagues (Uhler et al., 2002) we detected *Nkx6* expression in neuroblasts 2-2, 3-1, 3-2 and 4-2 (Fig. 5D). We also found *Nkx6* expression in neuroblasts 1-1 and 1-2 (Fig. 5E,F). By early stage 11, *Nkx6* was downregulated in neuroblasts and expressed in ganglion mother cells (GMCs) and postmitotic neurons. From stage 14 to the end of gastrulation, *Nkx6* was expressed in a segmentally reiterated pattern of CNS neurons (Fig. 5C). At stage 14, many of these Nkx6-positive cells also expressed the postmitotic motoneuron marker, pMAD (Marques et al., 2002) (Fig. 5H) suggesting that many, perhaps all, motoneurons are initially Nkx6-positive. However, at later stages Eve-positive motoneurons no longer expressed Nkx6 (Fig. 5G), consistent with the observation



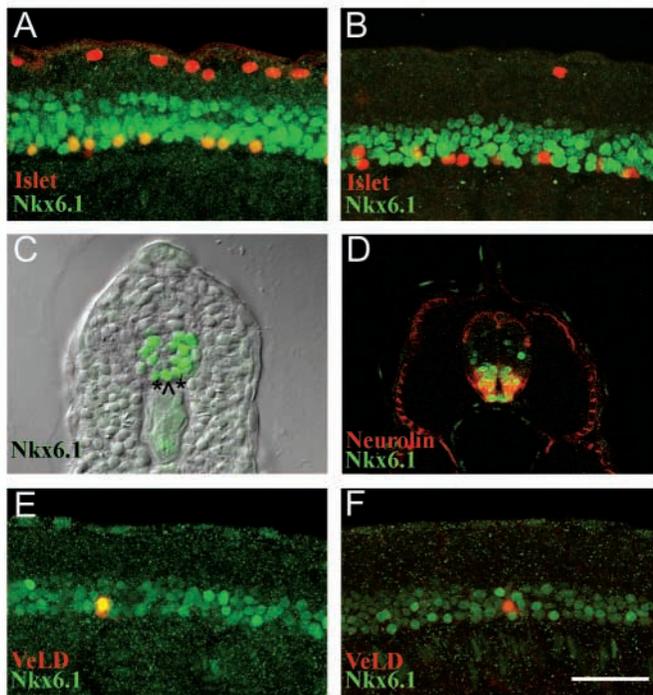
**Fig. 3.** *nkx6.1* is expressed in zebrafish ventral spinal cord.

(A) Expression is initiated in the embryonic shield at 50% epiboly, approximately 5 hpf (animal pole view, optical section of embryo without yolk). Asterisk denotes epiblast (outer) layer. (B) By bud stage, ~10 hpf, *nkx6.1* is broadly expressed in medial neuroectoderm (dorsal view, rostral to left). (C) At 26 hpf, *nkx6.1* is strongly expressed in ventral CNS from the midbrain through the tail (lateral view; rostral to left). Arrowhead denotes pancreas expression. (D) Higher magnification view of spinal cord shown in (C). Scale bar: 50  $\mu$ m for A,B,D; 25  $\mu$ m for C. sc, spinal cord; n, notochord; y, yolk.

that the CNS contained Nkx6-negative, pMAD-positive motoneurons (Fig. 5I). These results reveal that some Nkx6-positive motoneurons are derived from Nkx6-positive neuroblasts, and raise the possibility that other Nkx6-positive motoneurons are derived from Nkx6-negative neuroblasts. Therefore, it is likely that Nkx6 expression is differentially regulated in neuroblasts and motoneurons. These results also suggest that Eve-positive fly motoneurons are similar to fish PMNs in that they both downregulate Nkx6 expression during development.

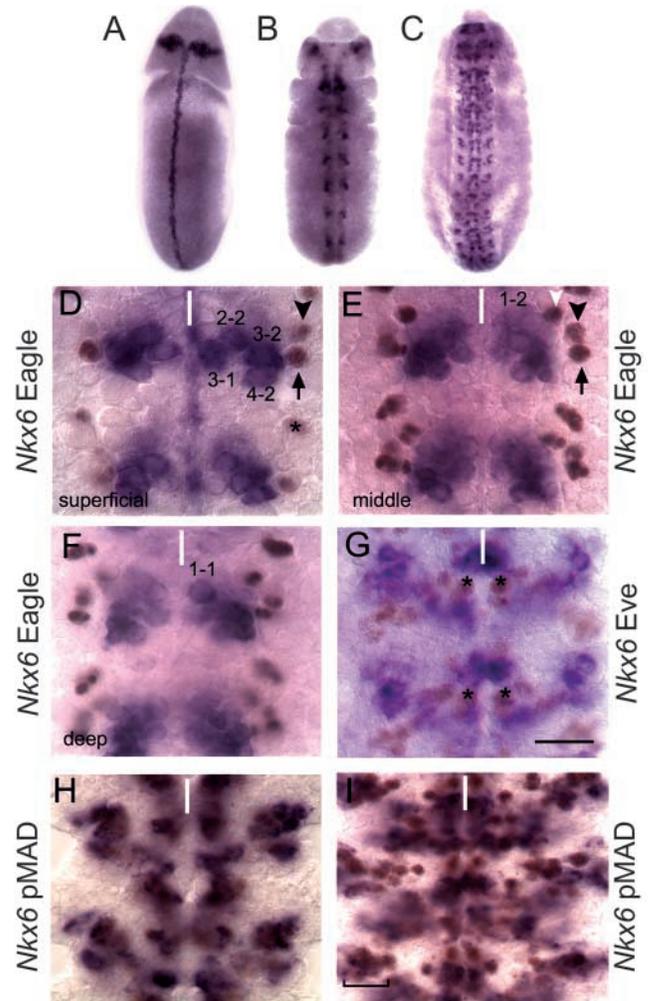
### Hh induces *nkx6.1* expression in zebrafish but not in flies

Studies in chick and mouse suggest Hh induces expression of Nkx6.1 in ventral neural tube (Briscoe et al., 2000), thus, we investigated whether Hh establishes or maintains *nkx6.1* expression in zebrafish. Injection of synthetic *shh* mRNA caused a dramatic dorsal expansion of *nkx6.1* expression. At 3-5 somites and 18 hpf, injected animals had many ectopic

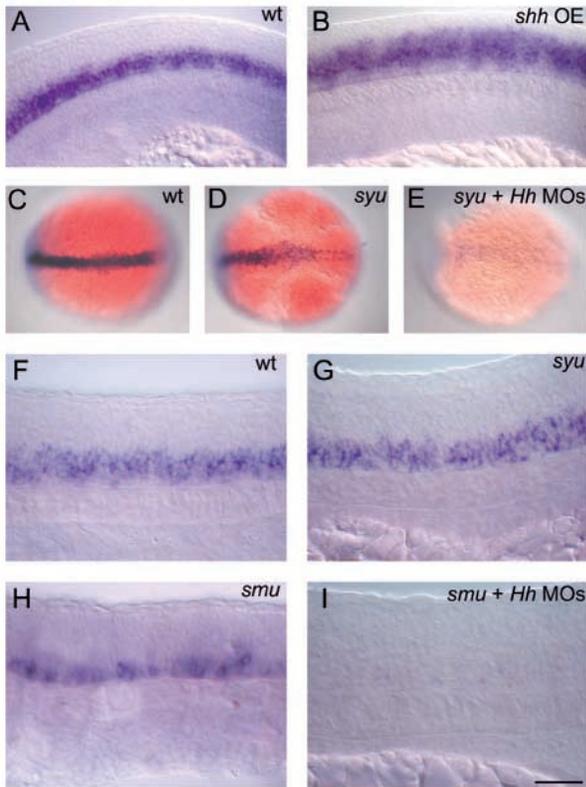


**Fig. 4.** Nkx6.1 is expressed in zebrafish motoneurons. Nkx6.1 (green) Islet1/2 (red) antibody double labels. (A) 14 hpf lateral view reveals Rohon-Beard neurons (RBs) in dorsal spinal cord express only Islet proteins and PMNs in ventral spinal cord are double labeled (yellow). (B) By 18 hpf Nkx6.1 is largely absent from PMNs; most RBs are out of focal plane. (C) Cross-section of 24 hpf embryo showing Nkx6.1-positive nuclei, which comprise about five cell rows in the ventral spinal cord including medial ( $\wedge$ ) and lateral ( $\ast$ ) floorplate. (D) Cross-section of 48 hpf embryo double labeled with Nkx6.1 (green) and Neuroilin (red) antibodies. Nkx6.1-positive nuclei are surrounded by Neuroilin-labeled cell surfaces. Laterally-located slow muscle cells are also Neuroilin-positive. (E) 20 hpf lateral view of individually labeled VeLD interneuron (yellow) expressing Nkx6.1 (green). Most individually labeled VeLDs in 20-22 hpf embryos expressed Nkx6.1 ( $n=11$ , 82%). (F) By 24 hpf VeLDs have downregulated Nkx6.1 ( $n=13$ , 85%). Scale bar: 50  $\mu$ m for A,B,D,E,F; 75  $\mu$ m for C.

*nkx6.1*-positive cells within the neural tube (Fig. 6B and data not shown) compared to wild types (Fig. 6A). Thus, Hh is sufficient to induce *nkx6.1* expression in zebrafish CNS. However, *nkx6.1* expression never expanded into the dorsalmost region of the neural tube.



**Fig. 5.** Nkx6 is expressed in fly ventral CNS and motoneurons. (A) Ventral view of stage 9 embryo showing Nkx6 RNA in cephalic neuroectoderm and CNS midline. (B) At stage 10, Nkx6 is ventrally restricted to rostral neuroblasts in each hemisegment. (C) By the end of gastrulation Nkx6 is expressed in neurons throughout the CNS. (D-F) Nkx6 is expressed in neuroblasts 1-1, 1-2, 2-2, 3-1, 3-2 and 4-2. Neuroblasts expressing Nkx6 mRNA (blue) were identified by their position relative to Eagle-positive neuroblasts (brown; NB2-4, arrowhead; NB3-3, arrow; NB6-4, asterisk; NB7-3, white arrowhead) and other neuroblasts. The same two segments of a stage 11 embryo are shown at different focal planes (D, superficial; E, middle; F, deep). White line, ventral midline. (G) At stage 14, Nkx6 (RNA in situ, purple) is confined to a population of neurons distinct from those expressing the dorsal motoneuron determinant Eve (antibody, brown). The prominent brown cells near the midline are RP2 motoneurons ( $\ast$ ). (H) pMAD (antibody, brown) and Nkx6 (RNA in situ, purple) expression are coincident in many cells at stage 14. (I) By stage 16, cells positioned laterally are positive for pMAD and Nkx6 (bracket), but more medial cells are clearly only pMAD-positive. Rostral is up in all images. Scale bar: 50  $\mu$ m for A-C; 16  $\mu$ m for D-I.



**Fig. 6.** Hh signaling induces zebrafish *nkx6.1* expression. (A) 18 hpf wild-type expression of *nkx6.1*. (B) Dramatic dorsal expansion of *nkx6.1* in an 18 hpf embryo ectopically expressing *shh* (*shh* OE, 87% of injected animals,  $n=283$ ). (C) 12 hpf wild-type expression of *nkx6.1*. (D) 12 hpf *syu* mutant with reduced *nkx6.1* expression. (E) 12 hpf *syu* mutant injected with *ehh* and *twhh* MOs. *nkx6.1* expression is largely absent (28% of injected clutch from *syu* heterozygous parents,  $n=120$ ). (F) Wild-type expression of *nkx6.1* in spinal cord at 24 hpf. (G) 24 hpf *syu* mutant has wild-type levels of *nkx6.1* expression in spinal cord. (H) Few *nkx6.1*-positive cells remain in a 24 hpf *smu* mutant spinal cord. (I) 24 hpf *syu* mutant injected with *ehh* and *twhh* MOs has no *nkx6.1* expression in spinal cord (21% of injected clutch from *syu* heterozygous parents,  $n=128$ ). C-D dorsal views; all others are lateral. Rostral to left in all images. Scale bar: 50  $\mu$ m for A,B; 100  $\mu$ m for C-E; 25  $\mu$ m for F-I.

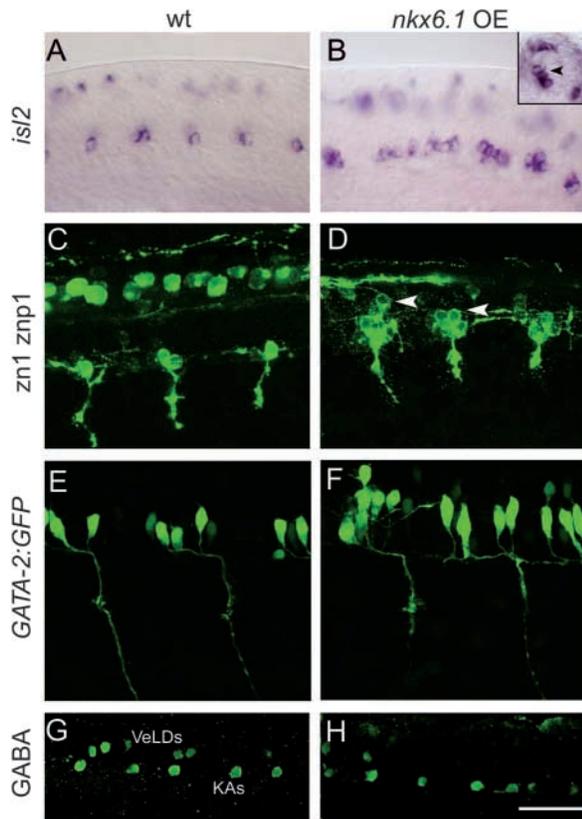
We next tested whether Hh is necessary for *nkx6.1* expression. At 24 hpf, Nkx6.1 expression appeared fairly normal in *syu* (*shh*) mutants (Fig. 6G), but was greatly reduced in *smu* (*slow muscle omitted* which encodes zebrafish Smoothened) mutants (Fig. 6H) as compared to wild types (Fig. 6F). *smu* mutants are more severe than *syu* mutants, however they still retain some early Hh signaling (Chen et al., 2001; Varga et al., 2001). To further suppress Hh signaling we injected *echidna hedgehog* (*ehh*) (Egger et al., 1995) and *tiggy winkle hedgehog* (*twhh*) (Currie and Ingham, 1996) morpholino antisense oligonucleotides (MOs) into *syu* mutants. At 24 hpf nearly 25% of injected embryos displayed severely reduced *nkx6.1* neural tube expression (Fig. 6I). This probably represents the *syu* mutant class with the greatest loss of Hh signals. About 75% of injected embryos had weaker spinal cord *nkx6.1* expression than wild types, and thus probably constituted knockdown of *ehh* and *twhh* in a wild-

type or heterozygous *syu* background. Earlier, at 12 hpf, about 25% of a clutch of *syu* mutants injected with *ehh* and *twhh* MOs had dramatically reduced *nkx6.1* expression (Fig. 6E) as compared to wild types (Fig. 6C) or *syu* mutants (Fig. 6D). Some embryos had a more severe loss than others, but in no embryo was *nkx6.1* completely absent. From these data we infer that Hh signals are required early to induce at least the vast majority of *nkx6.1* expression and later for its maintenance. Our experiments suggest that zebrafish *nkx6.1* acts downstream of Hh signaling, as in chick and mouse.

Hh expression is very different in vertebrate and fly embryos (reviewed by Eisen, 1998). In vertebrates, Hh is expressed in axial neurectoderm (floorplate) and underlying axial mesoderm (notochord). In contrast, in fly neurectoderm Hh is expressed in segmentally reiterated stripes orthogonal to the CNS midline. These stripes also express Engrailed (Tabata et al., 1992; Mohler and Vani, 1992), a marker for row 6 and 7 neuroblasts (Broadus et al., 1995). Thus, the Hh expression domain is adjacent to the six Nkx6-positive neuroblasts, raising the possibility that Hh might regulate Nkx6 expression in these cells. To test this, we examined Nkx6 expression in Hh mutants, which lack neuroblasts in rows 2, 5 and 6 (Matsuzaki and Saigo, 1996), including Nkx6-positive neuroblast 2-2. The Nkx6 expression pattern in the remaining five Nkx6-positive neuroblasts was wild type (data not shown), suggesting that flies utilize a different mechanism from vertebrates to establish Nkx6 expression. We also assessed whether Hh was required for formation of motoneurons derived from an Nkx6-positive neuroblast by examining co-expression of HB9 and pMAD in the RP1,3,4,5 motoneuron progeny of neuroblast 3-1. We found no change in these motoneurons in homozygous *hh* mutant embryos (data not shown), suggesting that Hh signaling is unnecessary for their formation.

### Nkx6.1 is sufficient to generate supernumerary motoneurons and suppress interneurons in zebrafish

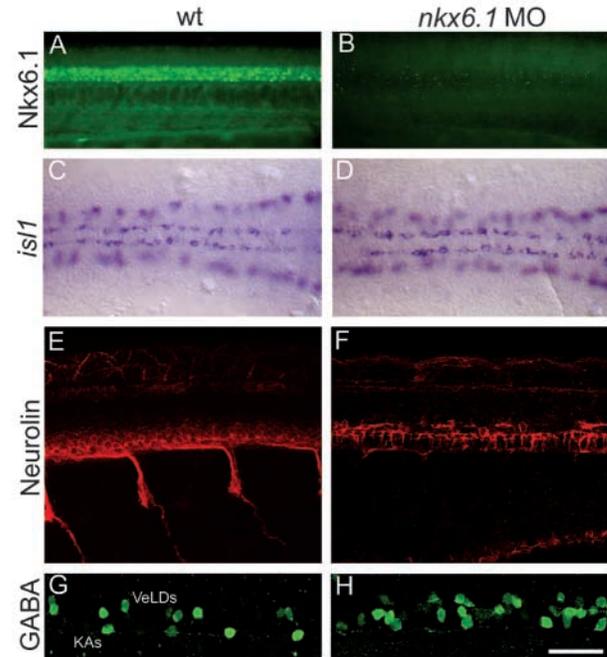
Because in zebrafish *nkx6.1* is expressed in motoneurons and their progenitors, we tested whether *nkx6.1* was sufficient to generate these cells. All zebrafish PMNs initially express *islet1* (*isl1*); later two specific PMNs, CaP and VaP downregulate *islet1* and initiate expression of a related gene, *islet2* (*isl2*) whereas two other PMNs, MiP and RoP continue to express *islet1* (Appel et al., 1998; Tokumoto et al., 1995). We injected synthetic *nkx6.1* mRNA and assayed for the presence of *islet1*-positive or *islet2*-positive PMNs by RNA in situ hybridization at 18 hpf. The *islet1* probe revealed supernumerary MiPs and RoPs (data not shown) and the *islet2* probe revealed supernumerary CaPs and VaPs (Fig. 7A,B), which we confirmed by labeling individual CaPs and VaPs in *nkx6.1*-injected embryos with fluorescent dextrans (data not shown). We also observed large clusters of zn1-positive motoneurons projecting axons into the periphery (Fig. 7D) as compared to wild types (Fig. 7C). Many of these supernumerary PMNs were located more dorsally than native PMNs (Fig. 7A-D), suggesting that Nkx6.1 converted some dorsal cells to a ventral fate. Injection of a *GATA-2:GFP* transgenic line that expresses GFP predominantly in SMNs (Meng et al., 1997) (Fig. 7E) with synthetic *nkx6.1* mRNA revealed supernumerary SMNs at 24 hpf (Fig. 7F). Because Nkx6.1 is expressed in VeLD interneurons (Fig. 4E,F), we also tested whether overexpression of Nkx6.1 affected this cell type.



**Fig. 7.** *nkx6.1* is sufficient to induce zebrafish motoneurons and repress interneurons. (A) *isl2*-positive CaPs and VaPs at 18 hpf. Wild-type embryos have  $1.3 \pm 0.7$  *isl2*-positive cells per spinal hemisegment ( $n=5$ ). (B) Embryos injected with *nkx6.1* mRNA (*Nkx6.1* OE) display supernumerary *isl2*-positive cells ( $2.3 \pm 0.5$  per spinal hemisegment;  $n=7$ ). These numbers are significantly different ( $P < 0.001$ ). Most RBs are not in focus in these images. Inset is a cross-section indicating the more dorsal position of supernumerary PMNs (arrowhead). (C) 24 hpf wild type labeled with zn1 and znp1 antibodies reveals 1 or 2 CaP/VaP cell bodies and axons as well as RBs. (D) Embryos injected with *nkx6.1* RNA have 3-4 CaP/VaPs with normal axons, but some are more dorsally located (arrowheads). RBs are out of focus. (E) Wild-type 24 hpf *GATA-2:GFP* embryo has clusters of  $3.0 \pm 1.7$  SMNs in each rostral spinal cord hemisegment (8 hemisegments in 2 embryos). (F) *nkx6.1* RNA-injected embryos have  $4.0 \pm 2.8$  SMNs per cluster ( $n=4$ ; numbers are significantly different,  $P < 0.009$ ). All views are lateral, rostral to left. Scale bar: 50  $\mu$ m for A,B; 33  $\mu$ m for C-H.

VeLDs are recognized by cell body position and GABA expression (Bernhardt et al., 1992). Embryos ectopically expressing *nkx6.1* RNA have fewer GABA-positive VeLDs than wild types at 24 hpf (Fig. 7G,H), suggesting cells that would normally become VeLDs become motoneurons instead.

Hh can induce *nkx6.1*, and *nkx6.1* is sufficient for formation of supernumerary PMNs and SMNs, suggesting that *nkx6.1* acts downstream of Hh. We tested this by injecting synthetic *nkx6.1* mRNA into clutches of embryos derived from *smu* heterozygotes; *smu* mutants have fewer PMNs and no SMNs because of reduced Hh signaling (Chen et al., 2001; Lewis and Eisen, 2001). At 18 hpf, approximately 25% of injected embryos lacked *isl2* expression in caudal spinal cord, just like 25% of embryos from a *smu* heterozygote cross (data not



**Fig. 8.** Nkx6.1 is required for zebrafish secondary motoneuron formation. (A) Nkx6.1 protein in 24 hpf wild-type spinal cord. (B) *nkx6.1* MO-injected embryo has no detectable protein in spinal cord at 24 hpf. (C) Wild-type and (D) MO-injected 12 hpf embryos have the same pattern of *isl1*-positive PMNs. RBs are out of focus on each side of PMNs. (E) At 48 hpf, ventral spinal cord is full of Neuroilin-positive SMNs. (F) MO-injected embryo has few remaining SMNs (75% of injected animals,  $n=565$ ). The brightly-stained cuboidal cells are floorplate. (G) GABA antibody labels KA and VeLD interneurons in ventral spinal cord at 24 hpf. (H) In MO-injected embryos there is a dramatic increase in GABA-positive VeLD interneurons ( $n=21$ ). Rostral to left in all images. A,B,E-H lateral views. C,D dorsal views. Scale bar: 50  $\mu$ m for A-D; 33  $\mu$ m for E-H.

shown), indicating that *nkx6.1* was insufficient to restore PMNs in the absence of Hh. Similarly at 30 hpf, *nkx6.1* was insufficient to restore SMNs in *smu* mutants (data not shown). We conclude that *nkx6.1* alone is only sufficient to induce motoneurons in the presence of Hh, suggesting that Nkx6.1 collaborates with additional factors downstream of Hh during motoneuron induction.

### Nkx6.1 is required for formation of zebrafish secondary motoneurons

To test whether Nkx6.1 is required for motoneuron formation, we injected embryos with an *nkx6.1*-specific MO (Fig. 8A,B and data not shown). Surprisingly, *nkx6.1* MO-injected animals had normal numbers of PMNs, revealed by *isl1* expression at 12 hpf and *isl2* expression at 18 hpf (Fig. 8C,D and data not shown). MO-injected embryos initiated the spontaneous tail reflex around 18 hpf, thus PMNs were functional (Saint-Amant and Drapeau, 1998). However, in contrast to embryos injected with a mispaired control *nkx6.1* MO which appeared wild type at 36 and 48 hpf, *nkx6.1* MO-injected embryos did not swim when touched, suggesting an absence of SMNs. Consistent with this, at 48 hpf, *nkx6.1* MO-injected animals consistently had fewer SMNs (Fig. 8F). At this stage there are more than

30 SMNs per spinal hemisegment; these cells are difficult to count because their somata are closely packed (Fig. 8E). Thus, we divided MO-injected embryos into two categories: those with nearly wild-type numbers of SMNs, and those with less than half of the wild-type number. Most MO-injected embryos had less than half of the wild-type number of SMNs (Fig. 8F); in a few cases SMNs were entirely absent. To test whether SMNs were dying in MO-injected embryos, we performed TUNEL assays at several stages between 18 and 48 hpf. There was no discernible difference in the number of TUNEL-positive nuclei in the ventral spinal cords of MO-injected and wild-type embryos at any stage (data not shown). We also tested whether decreased proliferation accounted for the decrease in SMNs in MO-injected embryos. BrdU incorporation showed no difference between MO-injected and wild-type embryos at 24, 30 and 36 hpf (data not shown).

We conclude that lack of SMNs is not due to a change in birth or death of these cells, suggesting that in the absence of Nkx6.1, they undergo a fate change. There are many more GABA-positive cells in the VeLD position (Fig. 8H) in *nkx6.1* MO-injected embryos than in wild-type embryos (Fig. 8G), suggesting that in the absence of Nkx6.1, SMNs develop as

VeLD interneurons. This phenotype is the opposite of that of embryos overexpressing Nkx6.1, supporting this hypothesis.

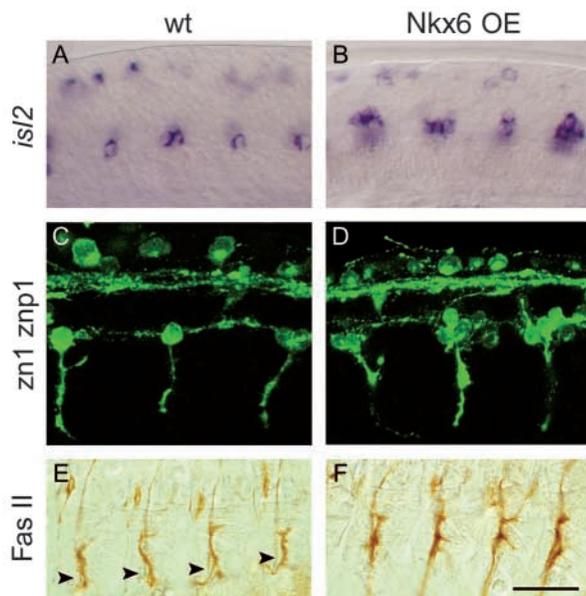
### Zebrafish and fly Nkx6 gene function is conserved in CNS patterning

We addressed whether Nkx6 genes have conserved functions by overexpressing the fly gene in zebrafish and the zebrafish gene in flies. We first asked whether ectopic expression of fly or zebrafish *Nkx6* produces the same phenotype in zebrafish embryos. 18 hpf zebrafish embryos overexpressing fly *Nkx6* mRNA had supernumerary PMNs (Fig. 9B,D) as compared to wild types (Fig. 9A,C), similar to the phenotype of embryos overexpressing zebrafish *nkx6.1* (Fig. 7); both fish and fly *Nkx6* appear equally potent at generating ectopic PMNs in zebrafish.

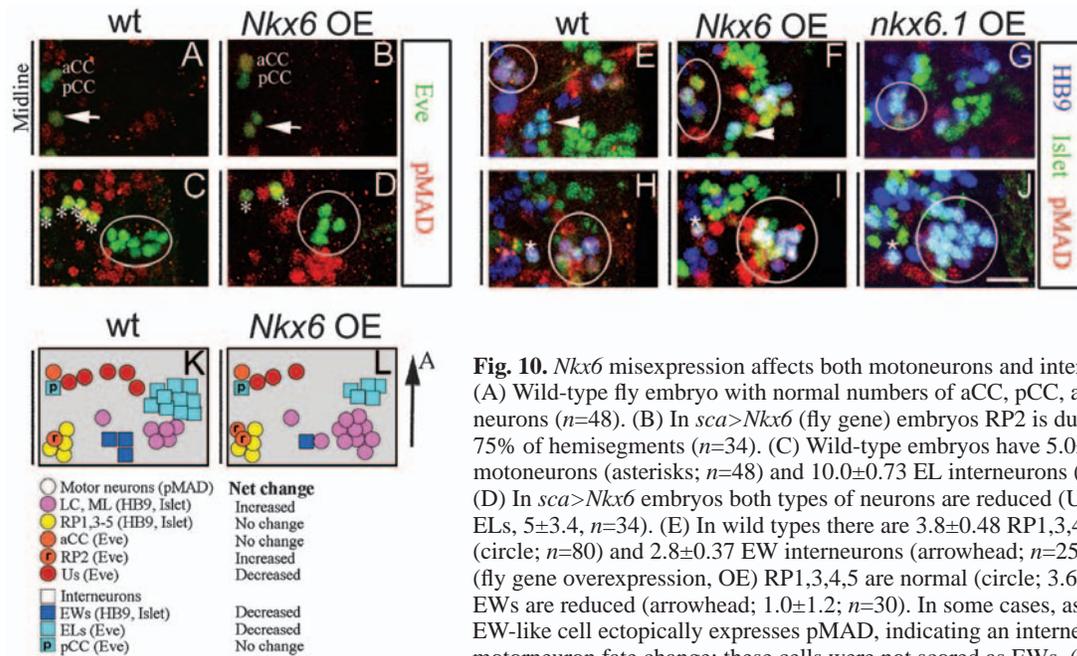
Next we tested whether zebrafish or fly *Nkx6* is sufficient to generate ectopic motoneurons in fly embryos. We created fly lines carrying UAS-*nkx6.1* (zebrafish) or UAS-*Nkx6* (fly) transgenes and used *sca-Gal4* to drive Nkx6 expression in neuroectoderm and all neuroblasts. Endogenous *Nkx6* is extinguished from neuroblasts by stage 12 (Fig. 5B); in contrast, in embryos expressing *sca-Gal4* and either the fly or the zebrafish transgene, Nkx6 expression is maintained at least through stage 13 (data not shown). We assayed for supernumerary motoneurons by molecular markers and motor projections. The segmental nerve B (SNb) motor nerve to ventral muscles was substantially thicker than in wild-type embryos (Fig. 9E,F), consistent with production of supernumerary motoneurons.

We next assayed embryos misexpressing fly or zebrafish Nkx6 genes for changes in several motoneuron markers: Eve, which labels all dorsally projecting motoneurons, Islet and HB9, which label ventrally projecting motoneurons, and pMAD, a pan-motoneuron marker (Broihier et al., 2002; Landgraf et al., 1999; Marques et al., 2002; Odden et al., 2002). Misexpression of either the fly or zebrafish gene resulted in supernumerary motoneurons and loss of interneurons in the fly CNS. Most supernumerary motoneurons were in the lateral cluster of HB9-positive, Islet-positive motoneurons (Fig. 10H,J). There was also occasional duplication of the Eve-positive RP2 motoneuron (Fig. 10A,B). We conclude that *Nkx6* is sufficient for formation of both ventrally projecting and dorsally projecting motoneurons. However, the phenotype of these embryos is complex. Some motoneurons appeared unaffected, for example the HB9-positive, Islet-positive RP1,3,4,5 motoneurons (Fig. 10E-G) and one type was slightly decreased, the Eve-positive U motoneurons (Fig. 10C,D). We also saw consistent loss of identified interneurons, including the Eve-positive ELs and Islet-positive EWs (Fig. 10C-F). Interestingly, in transgenic animals, cells in the EW position often expressed pMAD, a definitive motoneuron marker, consistent with a transformation of these interneurons into motoneurons.

Supernumerary motoneurons might arise from neuroblast duplication or change within a neuroblast lineage. To test whether there were duplicated neuroblasts, we examined *sca>Nkx6* or *sca>nkx6.1* fly embryos using various markers including Engrailed, Odd-skipped, Vnd, and Ind (McDonald et al., 1998; Patel et al., 1989; Spana and Doe, 1995; Weiss et al., 1998). We saw normal numbers of neuroblasts in both backgrounds (data not shown), ruling out neuroblast duplication, and suggesting that ectopic motoneurons result from an alteration in neuroblast lineage, for example, an



**Fig. 9.** Nkx6 function is conserved between fish and flies. (A) *isl2*-positive CaPs and VaPs in an 18 hpf zebrafish embryo. *isl2*-positive RBs are out of focus. Wild-type embryos have  $1.3 \pm 0.7$  *isl2*-positive motoneurons per spinal hemisegment ( $n=5$ ). (B) 18 hpf zebrafish embryo ectopically expressing *Nkx6* (fly gene, *Nkx6* OE) RNA with supernumerary *isl2*-positive cells ( $2.3 \pm 0.3$  *isl2*-positive cells per hemisegment), similar to the phenotype caused by ectopic expression of zebrafish *nkx6.1* RNA (compare with Fig. 7B). The number of *isl2*-positive motoneurons is significantly different ( $P < 0.0003$ ). (C) PMNs in 24 hpf wild-type embryo labeled with *zn1* and *znp1*; RBs and several axon tracts are also visible. (D) 24 hpf zebrafish embryo ectopically expressing *Nkx6* (fly gene) RNA generates ectopic PMNs (compare with Fig. 7D). (E) Stage 17 wild-type fly embryo stained with Fas II. Arrowheads indicate SNb nerves. (F) *sca>Nkx6.1* (fish gene) fly embryos with visibly thicker SNb nerves (88%,  $n=42$  hemisegments). (A-D) Lateral views of zebrafish spinal cord. (E,F) Dissected fly embryo musculature. Scale bar: 50  $\mu$ m for A,B; 33  $\mu$ m for C-F.



**Fig. 10.** *Nkx6* misexpression affects both motoneurons and interneurons in flies. (A) Wild-type fly embryo with normal numbers of aCC, pCC, and RP2 (arrow) neurons ( $n=48$ ). (B) In *sca>Nkx6* (fly gene) embryos RP2 is duplicated (arrow) in 75% of hemisegments ( $n=34$ ). (C) Wild-type embryos have  $5.0\pm 0.14$  U motoneurons (asterisks;  $n=48$ ) and  $10.0\pm 0.73$  EL interneurons (circle;  $n=46$ ). (D) In *sca>Nkx6* embryos both types of neurons are reduced (Us,  $2.5\pm 1.4$ ,  $n=34$ ; ELs,  $5\pm 3.4$ ,  $n=34$ ). (E) In wild types there are  $3.8\pm 0.48$  RP1,3,4,5 motoneurons (circle;  $n=80$ ) and  $2.8\pm 0.37$  EW interneurons (arrowhead;  $n=25$ ). (F) In *sca>Nkx6* (fly gene overexpression, OE) RP1,3,4,5 are normal (circle;  $3.6\pm 0.65$ ;  $n=73$ ) and EWs are reduced (arrowhead;  $1.0\pm 1.2$ ;  $n=30$ ). In some cases, as shown here, an EW-like cell ectopically expresses pMAD, indicating an interneuron-to-motoneuron fate change; these cells were not scored as EWs. (G) In *sca>nkx6.1* (fish gene) RP1,3,4,5 numbers are normal (circle,  $3.5\pm 0.66$ ;  $n=55$ ). (H) Wild-type

embryos have one medial lateral motoneuron (ML) per hemisegment (asterisk;  $80\pm 0$ ;  $n=80$ ) and  $4.7\pm 0.54$  HB9-, Islet-, pMAD-positive lateral cluster motoneurons (LC; circle;  $n=50$ ). (I) *sca>Nkx6* (fly gene) embryos have one ML motoneuron (asterisk; 94% of hemisegments;  $n=44$ ) and  $8.5\pm 2.3$  LC motoneurons ( $n=80$ ). (J) *sca>nkx6.1* (fish gene) also have one ML motoneuron (asterisk, 89.4% of hemisegments;  $n=57$ ) and  $8.0\pm 1.89$  LC motoneurons; ( $n=60$ ). (K) Schematic depicting average wild-type number of assayed motoneurons and interneurons. (L) Schematic depicting average number of motoneurons and interneurons in *sca>Nkx6* (fly gene) embryos. Scale bar: 15  $\mu$ m. All images are ventral views; rostral is up, medial is left and lateral is right.

interneuron to motoneuron transformation or a switch in GMC identity.

To examine potential lineage effects, we expressed fly *Nkx6* under the control of Eagle (Eg), which is expressed in neuroblast 7-3 and its progeny, the HB9-positive EW interneurons and GW motoneuron. We found the same number of Eg-positive, HB9-positive cells in controls and embryos overexpressing *Nkx6* ( $2.65\pm 0.69$  in 26 control hemisegments and  $2.64\pm 0.53$  in 42 hemisegments overexpressing *Nkx6*;  $P<0.34$ ). However, more than twice as many of these cells expressed pMAD in embryos overexpressing *Nkx6* than in controls ( $0.85\pm 0.83$  in 26 control hemisegments;  $1.93\pm 0.81$  in 42 hemisegments overexpressing *Nkx6*;  $P<0.002$ ) revealing that at least in the case of neuroblast 7-3 the supernumerary motoneurons arise within the lineage, presumably by changing EW interneurons into motoneurons.

We also tested whether *Nkx6* was necessary for fly motoneuron formation by RNAi. We saw no change in the numbers of HB9 or pMAD-positive cells in embryos lacking *Nkx6*, suggesting that it is not required for motoneuron formation (data not shown), consistent with the phenotype of *Nkx6* mutants (Broihier et al., 2004).

## Discussion

Our key finding is that *Nkx6* genes have conserved expression patterns and functions in flies and zebrafish. One function of *Nkx6* genes is to promote motoneuron and inhibit interneuron formation, however, as we discuss below, the role of this gene in neurogenesis is clearly more complex.

## Zebrafish *Nkx6.1* promotes motoneuron and suppresses interneuron formation

*Nkx6.1* is expressed in a zebrafish spinal cord domain that generates diverse cell types, including PMNs and SMNs, interneurons and oligodendrocytes (Park et al., 2002) (H. C. Park and B. Appel, personal communication). We focused on the role of *nkx6.1* in motoneurons and VeLD interneurons. However, a complete understanding of how this gene functions requires lineage analysis to ascertain when it is expressed in each cell type generated in this region, and how these cell types are related to one another. In zebrafish, *Nkx6.1* is required for formation of SMNs, but not PMNs. This is surprising considering that in mice lacking *Nkx6.1* all subtypes of spinal motoneurons are similarly depleted (Sander et al., 2000). Thus, we expected to see fewer PMNs and SMNs in zebrafish. It would be interesting to learn whether mice lacking *Nkx6.1* are missing specific motoneuron subsets, such as later-generated motoneurons. *Nkx6.1* is one of the few known proteins that differentially affects SMN and PMN formation in zebrafish, raising the possibility that other, related genes may participate in PMN formation. A good candidate is *Nkx6.2*, which is able to substitute for *Nkx6.1* in mice lacking *Nkx6.1* function (Vallstedt et al., 2001). Interestingly, mouse *Nkx6.2* is not normally expressed in the motoneuron progenitor domain, but its expression expands into this domain in the absence of *Nkx6.1*. It will be important to learn whether zebrafish has an *Nkx6.2* homolog, where it is expressed and if it is required for PMN formation.

The decreased number of SMNs in *nkx6.1* MO embryos was accompanied by an increase in VeLD interneurons. Conversely,

embryos ectopically expressing *nkx6.1* had fewer VeLDs. The simplest interpretation of these results is that when Nkx6.1 is present, ventral cells become SMNs and when it is absent, they become interneurons. However, we cannot rule out the possibility that VeLDs remain in the absence of Nkx6.1, but no longer express GABA and thus are not apparent in our assay. This could be resolved by developing a more specific probe for VeLDs that would allow them to be observed at a variety of developmental stages in wild-type and mutant embryos. Despite the need for further study in zebrafish, we favor the idea that Nkx6.1 promotes a motoneuron program and represses the VeLD interneuron program because such a role would be consistent with the function of Nkx6.1 in mouse ventral spinal cord (Sander et al., 2000; Vallstedt et al., 2001). It will be interesting to learn whether other interneurons from the same domain of the zebrafish spinal cord are similarly regulated.

### **Fly *Nkx6* generally promotes motoneuron and suppresses interneuron formation**

Overexpression of fly *Nkx6* in fly CNS produced a complex phenotype that might result from fate changes within neuroblast lineages. For example, cells in the EW interneuron position often expressed pMAD, a definitive motoneuron marker, suggesting an interneuron-to-motoneuron fate change. There are many more motoneurons in the lateral cluster than can be simply explained by EWs becoming motoneurons; thus other sources must also contribute to formation of supernumerary, HB9-positive, lateral cluster motoneurons. HB9-positive motoneurons project to ventral muscles via SNb (Broihier et al., 2002; Odden et al., 2002), thus, they probably contribute to the thicker SNb motor nerves. Both HB9 and Islet are required for proper ventral motoneuron projections (Broihier and Skeath, 2002; Odden et al., 2002; Thor and Thomas, 1997), suggesting Nkx6 interacts with them during this process.

Nkx6 and Eve are expressed in mutually exclusive neuronal subpopulations at the end of fly gastrulation. All dorsally projecting motoneurons are Eve-positive (Landgraf et al., 1999) and thus Nkx6 must be restricted to interneurons or the ventrally projecting, Islet-positive, HB9-positive motoneurons at this developmental stage. However, at earlier stages Nkx6 is expressed in some neuroblasts that generate Eve-positive motoneurons and probably in those motoneurons themselves. Thus, in flies as in fish, Nkx6 expression is apparently downregulated in at least some motoneurons. However, Nkx6 must play a role in the generation of these cells as overexpression causes duplicated Eve-positive RP2 motoneurons and fewer Eve-positive U motoneurons. Understanding how expression of Eve and Nkx6 is regulated in these cells should elucidate when Nkx6 acts during motoneuron formation.

### **Are Nkx6 genes ancient regulators of motoneuron development?**

Several dorsoventral patterning genes are expressed in strikingly similar patterns in the developing CNS of vertebrates and flies, motivating us to test whether these genes have conserved functions. Mouse Nkx2.2 and Gsh-1, orthologs of fly Vnd and Ind, produced no phenotype when overexpressed in fly embryos, revealing that the vertebrate proteins do not function in the context of the other species (T.V.O.,

unpublished) and suggesting functional domains have become distinct over time. That zebrafish Nkx6.1 and fly Nkx6 have similar overexpression phenotypes suggests their functions in CNS patterning have been preserved over 800 million years of evolution. We propose the bilaterian ancestor utilized Nkx6 proteins both to pattern ventral CNS and to promote motoneuron and suppress interneuron development. In vertebrates, Nkx6.1 is ventrally expressed, consistent with the ventral origin of CNS motoneurons. In flies, Nkx6 is also initially ventrally restricted, although its expression is not strictly correlated with motoneuron-producing neuroblasts, which are distributed throughout the CNS dorsoventral axis. Later, fly Nkx6 is expressed at least transiently in many, perhaps all, developing motoneurons, thus it must be expressed in some motoneurons whose neuroblast antecedent was Nkx6-negative. This suggests that expression of Nkx6 is differentially regulated in neuroblasts and their progeny.

How flies generate motoneurons from all CNS dorsoventral levels and vertebrates from only ventral CNS remains a mystery. Thor and Thomas (Thor and Thomas, 2002) proposed that the common ancestor of vertebrates and arthropods had both dorsally and ventrally projecting motoneurons and that vertebrates lost the dorsally projecting subset because of constraints imposed by evolution of the notochord. This is consistent with the observation that the ventrally projecting motoneurons in both flies and vertebrates express the same transcription factors, Nkx6, Islet, HB9 and Lim3. The origin of motoneurons can be addressed by studying nervous system patterning in other protostome and deuterostome phyla to learn whether ventral restriction of motoneurons is a basal or derived characteristic.

### **Transcription factors that control motoneuron formation are regulated by different mechanisms in vertebrates and arthropods**

The inputs establishing *Nkx6* expression have apparently diverged between vertebrates and arthropods. In flies there are at least two spatially and temporally distinct phases of Nkx6 expression, early in ventral neuroblasts and later in neurons, only some of which are derived from Nkx6-positive neuroblasts. This has allowed us to ask whether the same signaling mechanism affects Nkx6 function at different times and in different cell types. Because Hh is required for Nkx6 expression in vertebrates, we asked whether Hh also affected Nkx6 in flies. We found that Hh does not affect *Nkx6* expression in fly neuroblasts, showing that it is not involved in establishing the early pattern. We also found that motoneuronal progeny of at least one Nkx6-positive neuroblast develop in the absence of Hh signaling, thus Hh is also probably not involved in establishing the later pattern of Nkx6 expression. What regulates formation of fly motoneurons is still an enigma.

In contrast to flies, Hh is required to induce *nkx6.1* in all vertebrates tested thus far, including zebrafish. Because motoneurons arise from progenitors in the ventral domain it is unclear whether Nkx6 is regulated differently in progenitors and their progeny. This could be tested by downregulating Hh at later developmental stages.

The ability of ectopic *nkx6.1* to induce supernumerary PMNs in zebrafish raises a conundrum. Hh is both necessary and sufficient for *nkx6.1* expression, however we and others have found that ectopic Hh is insufficient to induce

supernumerary PMNs except in the most rostral spinal cord (Hammerschmidt et al., 1996) (S.E.C. and J.S.E., unpublished). How can a downstream gene be sufficient to generate a particular cell type when the upstream gene that induces it is insufficient? One possibility is that Hh promotes expression of both positive and negative regulators of motoneuron formation. Normal levels of Hh lead to a balance between these negatively acting and positively acting downstream genes, and PMNs are formed. In contrast, excess Hh tips the balance in favor of negative regulators, thus preventing formation of supernumerary PMNs. Hh is known to induce other factors that regulate motoneuron formation such as *olig2*, which can only generate PMNs in concert with Hh (Park et al., 2002), showing that additional Hh-dependent factors are required; *nkx6.1* is a good candidate for fulfilling this role.

It is clear that although the same transcription factors regulate formation of ventrally projecting motoneurons in vertebrates and arthropods, these transcription factors are regulated by different mechanisms in these distinct taxa. It will be exciting to learn what regulates the Nkx6 pathway leading to motoneuron formation in flies and whether formation of dorsally projecting and of ventrally projecting motoneurons are regulated by the same mechanisms.

We thank José Campos-Ortega, Andre Quinkertz, Jim Skeath, Heather Broihier, Bruce Appel and Hae-Chul Park for generously sharing data before publication, John Postlethwait, Bruce Appel, Susan Bassham, William Jackman and Stephen Schneider for critical comments on earlier drafts of the manuscript, Joanne Odden, Bruce Draper, Kristin Wildermuth and Deanna Grant for technical assistance, Ole Madsen for the generous gift of Nkx6.1 antibody, David Kimelman and Thierry Lepage for the gastrula stage cDNA library, the University of Oregon Zebrafish Facility staff for fish husbandry and members of the Eisen and Doe labs for support. Supported by NSF DGE9972830 (SEC), NIH HD27056 (TVO, MJL, CQD), and NIH NS23915 (JSE), with special thanks to Gabrielle LeBlanc.

We dedicate this paper to our dear friend and colleague José Campos-Ortega.

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